



## Effect of manganese and calcium ions on the photoinduced water oxidation with photosynthesis organ grana from green plant

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### ABSTRACT

To develop the visible light-induced hydrogen and oxygen production based on the water photolysis, 2,6-dichloroindophenol (DCIP) reduction based on the photoinduced water oxidation with oxygen evolved centre (OEC) of PSII in photosynthesis organ, grana from *spinach* was studied. It noted that manganese–calcium (4Mn–Ca) cluster consisted of four manganese and one calcium ions in OEC, and the role and effect of manganese (III) and calcium ions on the DCIP reduction were investigated. By addition of manganese (III) acetate up to 80  $\mu\text{mol dm}^{-3}$ , the concentration of the reduced DCIP with irradiation was increased compared with the system in the absence of manganese (III) acetate. The activity of DCIP reduction in the presence of 80  $\mu\text{mol dm}^{-3}$  manganese (III) acetate was 4.7 times higher than that in the absence of manganese (III) acetate. This result shows that the DCIP photoreduction with grana was promoted by addition of manganese (III) ion. In contrast, the activity of DCIP reduction in the presence of 120  $\mu\text{mol dm}^{-3}$  manganese (III) acetate was 1.5 times higher than that in the absence of manganese (III) acetate, indicating that photoreduction of DCIP activity of grana was inhibited under higher concentration of manganese (III) ion or acetate anion. By addition of 80  $\mu\text{mol dm}^{-3}$  calcium chloride, on the other hand, 5.9  $\mu\text{mol dm}^{-3}$  of reduced DCIP was produced and the reduction ratio of the reduced DCIP to DCIP was 8.6% after 240 min irradiation. The activity of DCIP reduction in the presence of 80  $\mu\text{mol dm}^{-3}$  calcium chloride was almost the same in the absence of calcium chloride. This result shows the no effect on the DCIP photoreduction based on the water oxidation with grana by addition of calcium ion.

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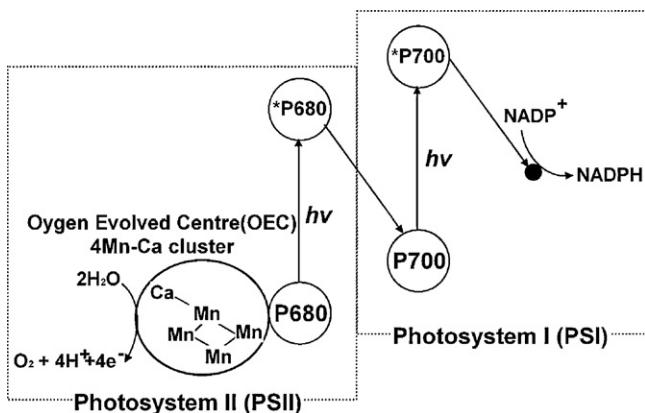
### 1. Introduction

Photoinduced hydrogen and oxygen production based on the water photolysis with natural and artificial photosynthesis has been studied extensively by means of converting solar energy to chemical energy [1–7]. In the viewpoint of new clean energy development, visible light-induced hydrogen production systems containing an electron donor (D), a photosensitizer (P), an electron relay (C), and a catalyst have been widely studied. In this system, colloidal platinum [5,6] and hydrogenase [3,4,7] are widely used as a catalyst. Colloidal platinum is stable against long-term visible light irradiation. Photosynthesis dye pigment, chlorophylls, which acts as the effective light-harvesting compound in photosynthesis of green plant, has absorption maximum around 430 and 670 nm [8]. Chlorophyll exhibits physiological functions as follows; the light-harvesting for the photolysis of water and the reduction of NADP<sup>+</sup>, and CO<sub>2</sub> fixation under visible light irradiation. Thus, the light-harvesting function of chlorophylls is attractive for the photoinduced hydrogen production system. We previously reported

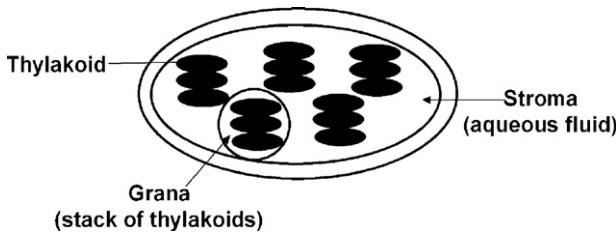
the photoinduced hydrogen production with the light-harvesting function of chlorophyll-*a* and colloidal platinum [9–12] and the effect of wavelength of light source on the photoinduced hydrogen production [11]. However, the development of water splitting into hydrogen and oxygen is not yet attained. Development of the optimal catalyst for oxidizing water especially attracts expectation. In natural photosynthesis of higher green plant and oxygenic photosynthetic cyanobacteria, photosynthesis reaction consists of two photosystems as shown in Fig. 1. One is the photosystem I (PSI) with reaction centre P700, which acts as a photoreduction of NADP<sup>+</sup>. The other one is the photosystem II (PSII) with reaction centre P680, which acts as an oxygen production based on the water oxidation. The photosystem II (PSII) in photosynthesis protein is attracting attention as an oxidation catalyst of water [13]. The oxygen evolved centre (OEC) contained in PSII works as an oxidation catalyst of water. Manganese–calcium cluster (4Mn–Ca) consisted of four manganese and one calcium ions in OEC is active site for water oxidation. The biophysical properties of 4Mn–Ca cluster have been studied widely [14–18]. It has been reported that OEC is only manganese cluster consisting of four manganese ions. However, it was shown clearly that OEC consisted of four manganese and one calcium ions, recently. The role of these metal ions on the water oxidation is not clarified yet. Thus, the effect of these metal ions on the

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**Fig. 1.** Outline of oxygenic photosynthesis reaction in higher green plant and cyanobacteria.



**Fig. 2.** Schematic representation of chloroplast.

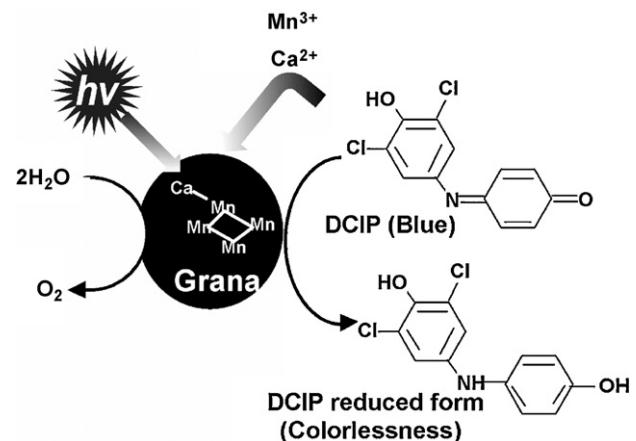
water oxidation activity of OEC is very important to clarify the role of manganese and calcium ions. As PSII purified from green plant such as *spinach* is unstable protein, however, it is difficult to investigate the addition effect of these ions on the water oxidation activity of OEC in PSII directly. Thus, we paid our attention to grana from green plant, which is stack of photosynthesis membrane thylakoids as shown in Fig. 2, are photosynthesis organ including photosystem II protein. As grana have stable water oxidation activity compared with purified PSII, it is suitable for investigating the addition effect of these ions on the water oxidation activity of OEC. The elucidation of the role of these ions in OEC leads to the development of biocatalyst based on grana with highly water oxidation activity. Moreover, we previously suggested the water photolysis system with grana [19,20]. The development of grana with highly water oxidation activity leads to the splitting of water into hydrogen and oxygen based on the photosynthesis reaction. We previously reported the effect of manganese (II) and (III) ions on the photoinduced water oxidation activity of grana from *spinach* as a short communication letter [21]. In contrast, the calcium ion in 4Mn–Ca also will act as an important role on the photoinduced water oxidation. However, the effect of manganese (III) and calcium ions on the photoinduced water oxidation activity of grana are not clarified yet.

In this study, to elucidate the role of manganese (III) and calcium ions in OEC and to develop the artificial photosynthesis system using grana with highly water oxidation activity, the effect of manganese (III) and calcium ions on the photoinduced water oxidation system with grana from *spinach* and 2,6-dichloroindophenol (DCIP) as an electron acceptor as shown in Scheme 1 was investigated.

## 2. Experimental

### 2.1. Materials

Sucrose, sodium chloride, magnesium chloride, potassium hydroxide, manganese (III) acetate and calcium chloride were obtained from Wako Chemical Co. Ltd. Tricine and  $\alpha$ - $\beta$ -



**Scheme 1.** DCIP photoreduction based on the water oxidation with grana. DCIP<sub>red</sub>: reduced form of DCIP.

octylglucoside were purchased from Dojin Laboratory Co. Ltd. 2,6-Dichloroindophenol (DCIP) and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) were purchased from Tokyo Kasei Co. Ltd. All the other reagents were higher grade available.

### 2.2. Isolation of grana from *spinach*

Grana were isolated from *spinach* according to the previous reported method [22]. At first, 100 g crushed leaf of *spinach* was homogenized in the solution containing 0.4 mol dm<sup>-3</sup> sucrose, 10 mmol dm<sup>-3</sup> potassium chloride and 2.0 mmol dm<sup>-3</sup> magnesium chloride in 200 ml of 50 mmol dm<sup>-3</sup> Tricine-KOH buffer (pH 8.0) with mechanical mixer. The homogenized sample was filtrated by the gauze. The filtrate was centrifuged at 5000 × g for 2 min at 5 °C and then grana were obtained as a precipitate. As grana were insoluble in aqueous solution, grana obtained were solubilized in 1.0%  $\alpha$ - $\beta$ -octylglucoside surfactant micellar solution. The chlorophyll-a and -b (Chl-a and -b) concentration in grana solution was measured using photodiode array UV-vis spectrophotometer (Scinco S-3100) as following method [23]. Grana were suspended into 200  $\mu$ l water and then 800  $\mu$ l acetone was added to the grana suspension. The sample mixture was centrifuged at 3000 × g for 5 min at room temperature. Chl-a and -b were included to liquid phase and the other components were precipitated as solid. The absorbance at 646.6 and 663.6 nm of liquid phase was measured using photodiode array UV-vis spectrophotometer. The molar coefficients of Chl-a and -b in water-acetone (1:4) mixture were 18.58 and 47.04 at 646.6, and 76.79 and 9.79 mmol<sup>-1</sup> dm<sup>-3</sup> cm<sup>-1</sup> at 663.6 nm, respectively. The observed absorbance at 646.6 ( $A_{646.6}$ ) and 663.6 nm ( $A_{663.6}$ ) were described as following equations:

$$A_{646.6} = 18.58[\text{Chl-}a] + 47.04[\text{Chl-}b] \quad (1)$$

$$A_{663.6} = 76.79[\text{Chl-}a] + 9.79[\text{Chl-}b] \quad (2)$$

Thus, the concentrations of Chl-a and -b were described as following equations:

$$[\text{Chl-}a] = 13.71 A_{663.6} - 2.85 A_{646.6} \quad (3)$$

$$[\text{Chl-}b] = 22.39 A_{646.6} - 5.42 A_{663.6} \quad (4)$$

Thus, the total chlorophyll concentration was indicated as Eq. (5):

$$[\text{Chlorophyll}] = 19.54 A_{646.6} + 8.29 A_{663.6} \quad (5)$$

The amount of manganese and calcium ions in grana solution is measured using an inductively coupled plasma-optical emission spectrometer (ICP-OES, Optima 3000DV, PerkinElmer).

### 2.3. Oxygen-evolved activity of grana

Photoinduced oxygen-evolution activity of grana was carried out a following method. The sample solution containing 0.6 ml grana (chlorophyll concentration:  $9.0 \text{ mmol dm}^{-3}$ , the number of 4Mn–Ca cluster:  $2.4 \times 10^{16}$ ,  $66 \mu\text{mol dm}^{-3}$ ),  $2.0 \text{ mmol dm}^{-3}$  DCIP, and  $0.42 \text{ mmol dm}^{-3}$  DCMU in 3.0 ml of phosphate buffer (pH 8.0) was deaerated by freeze–pump–thaw cycles for 6 times and then substituted by nitrogen gas. DCMU acts as a role of inhibitor of electron transfer between PSII and PSI. In this study, to use the function only of PSII in grana, it is necessary to inhibit the electron transfer process between PSII and PSI. Thus, DCMU was added in the reaction system. The sample solution is irradiated with 150 W halogen lamp at  $30^\circ\text{C}$ . The light of the wavelength less than 600 nm was removed by optical sharp cut-off filter. The amount of oxygen produced was determined by oxygen meter with Clark-type polarographic electrode (Strathkelvin Instruments Ltd., Model 482 and SI130 Microcathode Oxygen Electrode).

### 2.4. Photoreduction of DCIP

Photoreduction of DCIP based on the water oxidation with grana was carried out a following method. The sample solution containing 0.02 ml grana (chlorophyll concentration:  $0.33 \text{ mmol dm}^{-3}$ , the number of 4Mn–Ca cluster:  $8.8 \times 10^{15}$ ,  $4.9 \mu\text{mol dm}^{-3}$ ),  $66 \mu\text{mol dm}^{-3}$  DCIP,  $10 \mu\text{mol dm}^{-3}$  DCMU, manganese (III) acetate or calcium chloride in 3.0 ml of phosphate buffer (pH 8.0) was deaerated by freeze–pump–thaw cycles for 6 times, and substituted by argon gas. The sample solution was irradiated with 150 W halogen lamp at  $30^\circ\text{C}$ . The light of the wavelength less than 600 nm was removed by optical sharp cut-off filter. The concentration of reduced DCIP is determined by absorption at 603 nm (molar coefficient of DCIP:  $1.4 \times 10^4 \text{ mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}$ ) using spectrophotometer. The concentration of manganese (III) acetate or calcium chloride was changed between 0 and  $120 \mu\text{mol dm}^{-3}$ .

## 3. Results and discussion

### 3.1. Isolation of grana from *spinach*

At first, properties of grana from *spinach* solution were studied. To check the existence of the OEC consisting of manganese cluster in grana prepared, the amount of manganese ions in grana solution was measured using an IPC-OEM analysis. 36 and  $10 \mu\text{mol}$  of manganese and calcium ions were contained in 1 ml of grana solution. 4Mn–Ca cluster in OEC was consisted of four manganese ions and one calcium ion. From IPC-OEM analysis, the amount of manganese ion was ca. 3.6 times molar than that of calcium ion in 1 ml of grana solution. Thus, it was presumed that about  $5.4 \times 10^{17}$  4Mn–Ca cluster was contained in 1 ml of grana solution. Next, the amount of chlorophyll in grana solution was measured using spectrophotometer by Eq. (5). Fig. 3 shows the UV-vis absorption spectrum of grana solubilized in d- $\beta$ -octylglucoside solution. Absorption bands around 630–700 nm attributed to chlorophyll-a and -b were observed. By Eq. (5),  $1.5 \mu\text{mol}$  of chlorophyll (chlorophyll-a and -b) was contained in 1 ml of grana solution.

### 3.2. Oxygen-evolved activity of grana

Visible light-induced oxygen production with the system containing grana, DCIP, and DCMU was attempted. The time dependence of oxygen evolution is shown in Fig. 4. After 240 min irradiation,  $1.3 \times 10^{-7} \text{ mol}$  of oxygen was evolved. From IPC-OEM analysis, it was presumed that about  $2.4 \times 10^{16}$  ( $4.0 \times 10^{-8} \text{ mol}$ ) of 4Mn–Ca cluster was contained in 0.6 ml of grana solution. The

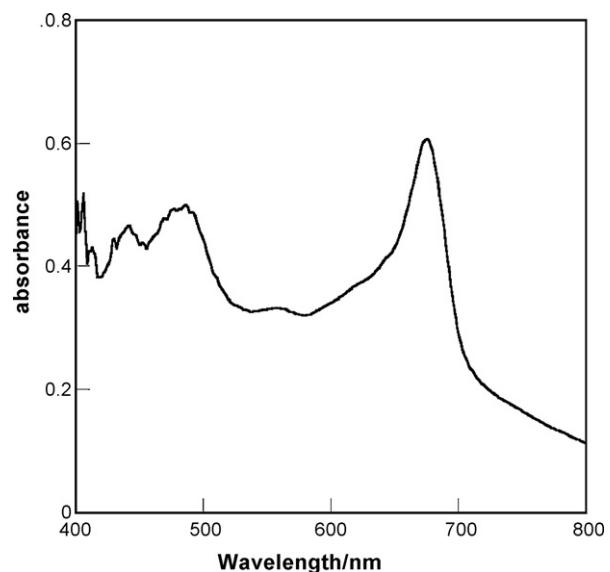


Fig. 3. UV-vis absorption spectrum of grana obtained from *spinach* with d- $\beta$ -octylglucoside micellar solution.

turnover frequency per hour ( $\text{TF h}^{-1}$ ) of 4Mn–Ca cluster in grana was defined as following equation:

$$\text{TF}(\text{h}^{-1}) = \frac{\text{amount of oxygen evolved}/\text{amount of 4Mn–Ca cluster}}{4(\text{h})} \quad (6)$$

Thus, the TF of 4Mn–Ca cluster in grana solution was estimated to be ca.  $0.4 \text{ h}^{-1}$ . In contrast, the no oxygen production was observed without irradiation (X). These results show that grana obtained from *spinach* have water oxidation activity based on the function of OEC.

### 3.3. Photoreduction of DCIP

Visible light-induced DCIP reduction based on the water photolysis with the function of PSII in grana was attempted. To attain the stable and effective oxygen production system, the effect of manganese (III) and calcium ions on the photoinduced water oxidation system was investigated. Fig. 5 shows the time dependence of reduced form of DCIP production with the system containing grana, DCIP, DCMU and metal ion (manganese (III) acetate or calcium chlo-

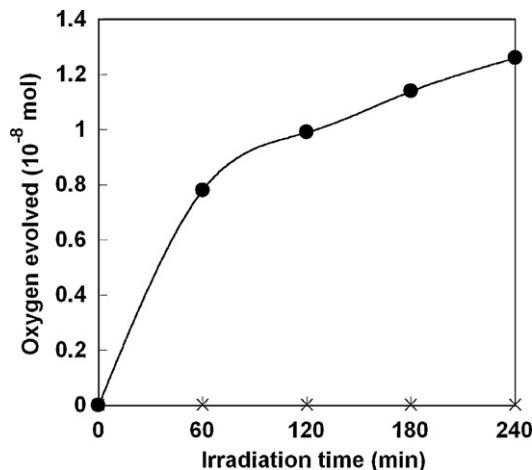
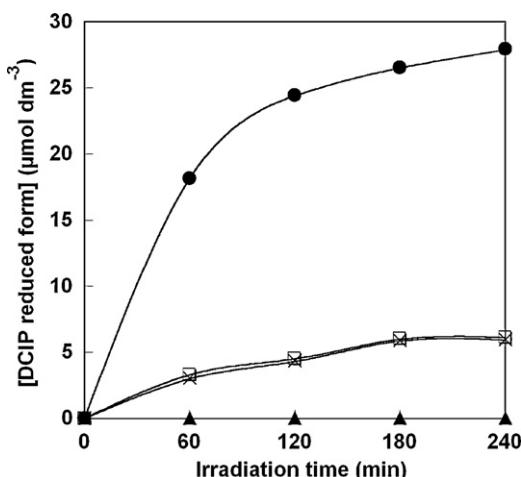


Fig. 4. Time dependence of photoinduced oxygen evolution with the system containing grana, DCIP and DCMU. X: dark condition.

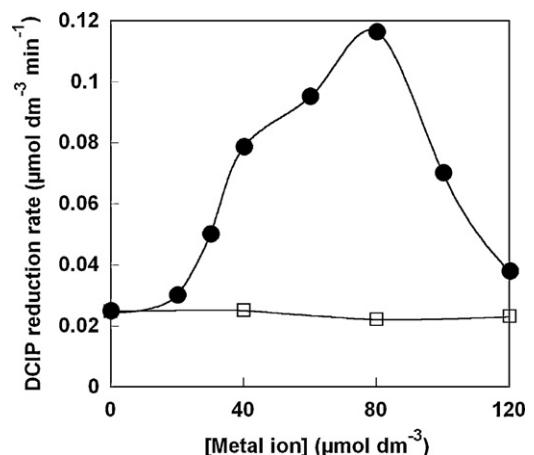
ride). In all cases, the DCIP was reduced with irradiation time. In contrast, no reduction of DCIP was observed without irradiation (closed triangle). Moreover, no reduction of DCIP also was observed in the system containing DCIP, DCMU and metal ion of manganese (III) or calcium ion (in the absence of grana; open circle). As there was no electron-donating reagent in the reaction system, DCIP was reduced with irradiation, indicating that water acts as an electron-donating reagent. The DCIP was not reduced with manganese (III) or calcium ion, directly. By addition of manganese (III) acetate, the concentration of the reduced DCIP with irradiation was increased compared with the system in the absence of manganese (III) acetate as shown in Fig. 5 (closed circle). By addition of 80  $\mu\text{mol dm}^{-3}$  manganese (III) acetate, 28  $\mu\text{mol dm}^{-3}$  of reduced DCIP was produced and the reduction ratio of the reduced DCIP to DCIP was 41% after 240 min irradiation. The TF per hour (TF  $\text{h}^{-1}$ ) of 4Mn–Ca cluster in grana was defined as following equation:

$$\text{TF}(\text{h}^{-1}) = \frac{[\text{reduced DCIP}]/[4\text{Mn–Ca cluster}]}{4(\text{h})} \quad (7)$$

By addition of 80  $\mu\text{mol dm}^{-3}$  manganese (III) acetate, the concentration of reduced DCIP was 28  $\mu\text{mol dm}^{-3}$  after 240 min irradiation. The concentration of 4Mn–Ca cluster in grana solution was 4.9  $\mu\text{mol dm}^{-3}$ . Thus, the TF of 4Mn–Ca cluster in grana solution was estimated to be *ca.* 1.4  $\text{h}^{-1}$ . In contrast, the TF of 4Mn–Ca cluster in grana solution in the absence of manganese (III) acetate was estimated to be *ca.* 0.3  $\text{h}^{-1}$ . The activity of DCIP reduction in the presence of 80  $\mu\text{mol dm}^{-3}$  manganese (III) acetate was 4.7 times higher than that in the absence of manganese (III) acetate. This result shows that the DCIP photoreduction with grana was promoted by addition of manganese (III) ion. In contrast, little change in DCIP photoreduction rate was observed by addition of calcium chloride (open square), compared with the system in the absence of calcium ion (X). By addition of 80  $\mu\text{mol dm}^{-3}$  calcium chloride, 5.9  $\mu\text{mol dm}^{-3}$  of reduced DCIP was produced and the reduction ratio of the reduced DCIP to DCIP was 8.6% after 240 min irradiation. This result shows that the DCIP photoreduction with grana was not affected by addition of calcium ion. As the phosphate buffer containing potassium and sodium ions was used as a solvent in these experiments, effects of these ion species on the DCIP photoreduction with grana were investigated. However, little change in DCIP photoreduction rate was observed by addition of sodium or potassium ion up to 120  $\mu\text{mol dm}^{-3}$ , compared with the system in the absence of ions. Thus, these ions includ-



**Fig. 5.** Time dependence of photoreduction of DCIP with the system containing grana, DCIP, DCMU and metal ion. Closed circle: 80  $\mu\text{mol dm}^{-3}$  manganese (III) acetate, open square: 80  $\mu\text{mol dm}^{-3}$  calcium chloride and X: absence of metal ion. Closed triangle: dark condition. Open circle: without grana.



**Fig. 6.** Relationship between concentration of metal ion and DCIP reduction rate. Closed circle: manganese (III) acetate; open square: calcium chloride.

ing phosphate buffer was not affected to DCIP photoreduction with grana.

#### 3.4. Effect of metal ion on photoreduction of DCIP

As mentioned above, the effect of metal ions on the photoreduction of DCIP with grana was studied and manganese ion acts as a promoter of water oxidation with grana. Next, the role and effect of metal ion concentration on the photoreduction of DCIP based on the water oxidation with grana were investigated. The relationship between the DCIP photoreduction rate and the concentration of metal ion is shown in Fig. 6. The photoreduction rate of DCIP increases with the concentration of manganese (III) ion up to 80  $\mu\text{mol dm}^{-3}$  and then decreases as shown in Fig. 6 (closed circle). These results show that the photoreduction of DCIP and the activity of oxygen-evolved complex were inhibited under higher concentration of manganese (III) ion. In contrast, the photoreduction rate of DCIP does not change with the concentration of calcium ion as shown in Fig. 6 (open square). These results show the no effect of calcium ion on the photoreduction of DCIP and the activity of oxygen-evolved complex. From these results, the possible mechanism of OEC is as follows. As the photoreduction of DCIP with the system containing grana, DCIP and DCMU was promoted by addition of manganese (III) ion, the site which consists of four manganese ions acts as a water oxidation catalyst. The increasing DCIP photoreduction activity of grana with the concentration of manganese (III) ion up to 80  $\mu\text{mol dm}^{-3}$  is due to the reconstruction of active site of manganese cluster structure by addition of manganese (III) ion. However, DCIP photoreduction activity of grana decreased by addition of excess manganese (III) acetate. In contrast, DCIP photoreduction activity of grana maintained by addition of excess manganese (III) chloride. In general, chloride ion is need to OEC activity. Thus, the reason of deactivation of grana is due to the chloride ion exchange by excess acetate anion. In contrast, the DCIP photoreduction with grana was not affected by addition of calcium ion. Thus, the contribution of calcium ion for water oxidation catalysis was little in OEC.

Next, let us focus on the DCIP reduction activity of grana in the presence of manganese (III) and calcium ions. The sample solution containing 0.02 ml grana, 66  $\mu\text{mol dm}^{-3}$  DCIP, 10  $\mu\text{mol dm}^{-3}$  DCMU, 80  $\mu\text{mol dm}^{-3}$  manganese (III) acetate and 80  $\mu\text{mol dm}^{-3}$  calcium chloride in 3.0 ml of phosphate buffer (pH 8.0) was irradiated with 150 W halogen lamp at 30 °C. The concentration of reduced DCIP was 27  $\mu\text{mol dm}^{-3}$  after 240 min irradiation. The concentration of reduced DCIP was almost the same that. Little contribution of calcium ion for water oxidation catalysis also was

observed in the presence of manganese (III) ion. Moreover, no deactivation of grana also was observed by addition of calcium ion. Thus, calcium ion will act as a role of stabilization of OEC structure.

#### 4. Conclusion

In conclusion, the photoreduction of DCIP with the system containing grana, DCIP and DCMU was promoted by addition of manganese (III) ion up to  $80 \mu\text{mol dm}^{-3}$ . On the other hand, the activity of DCIP reduction in the presence of  $80 \mu\text{mol dm}^{-3}$  calcium chloride was almost the same in the absence of calcium chloride, indicating the no effect on the DCIP photoreduction with grana by addition of calcium ion. The effect of addition of manganese (III) and calcium ions to an oxygen production was under progress now. Moreover, by using this system, the splitting of water into hydrogen and oxygen will be accomplished in the future.

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